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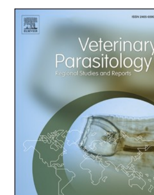

Original Article

Benzimidazole-resistance associated mutation in *Haemonchus contortus* in Norwegian sheep, as detected by droplet digital PCR

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ABSTRACT

The aim of this study was to investigate the occurrence of benzimidazole-resistant *Haemonchus contortus* in Norwegian sheep flocks. Screening was based on detection of one of the resistance-conferring mutations in the β tubulin isotype 1 gene (F200Y, TAC) in larvae (L3) cultivated from *H. contortus* eggs from naturally infected sheep. Faecal samples were collected in 2021/2022 from flocks in the northern ($n = 34$), central ($n = 7$), eastern ($n = 40$), southern ($n = 1$), and western ($n = 87$) areas of Norway. In total, samples were taken from 169 flocks (spring-ewes samples: 167, autumn-lambs samples: 134). Individual faecal samples were collected from 10 randomly selected ewes (spring) and 10 randomly selected lambs (autumn) in each flock. Faecal samples collected from each flock on each occasion were pooled (lamb and ewe samples pooled separately) and cultured for L3 development. After harvest of larvae (Baermann method), DNA was extracted and then analysed using droplet digital PCR with primer/probe sets targeting the BZ-associated F200Y (TAC) mutation.

Haemonchus was found in 60% (80/134) of samples from lambs, and in 63% (106/167) from ewes. Among these, the F200Y mutation was detected in 73% (58/80) of larval samples from lambs and 69% (73/106) of larval samples from ewes, respectively. Although regional differences were evident, the mutation was detected in all areas indicating a widespread distribution and a strong potential for an increasing problem with treatment-resistant haemonchosis in Norwegian sheep flocks.

1. Introduction

Sheep are always infected with parasitic worms when grazing on pasture. One of the most pathogenic worms is the bloodsucking nematode, *Haemonchus contortus.* This parasite is distributed worldwide but prefers warm and humid climates (Morgan and van Dijk, 2012; Rinaldi et al., 2015; Santos et al., 2012). The current main solution for control of this parasite is anthelmintic treatments, of which benzimidazoles (BZ) are the most frequently used drug class in Norway (Domke et al., 2011; Gravdal et al., 2021).

The development of anthelmintic resistance (AR) among gastrointestinal nematodes (GIN) in farmed ruminants is widespread in Europe (Rose et al., 2015), including BZ-resistant *H. contortus* reported from Sweden (Höglund et al., 2022, 2009), the Netherlands (Borgsteede et al., 2007), Belgium (Claerebout et al., 2020), Germany and Switzerland (Scheuerle et al., 2009) to name but a few. This resistance has also already been detected in sheep flocks in the south-western region of Norway, through the faecal egg count reduction test (FECRT) (Domke et al., 2012). It is well established that AR can lead to production losses (Miller et al., 2012) and poses a risk to animal health and welfare (Coles and Roush, 1992).

Several polymerase chain reaction (PCR)-based techniques have been developed to detect BZ resistance in GIN of ruminants (Alvarez-Sánchez et al., 2005; Elard et al., 1999; Humbert et al., 2001; Silvestre and Humbert, 2000). In the 1990s, a single nucleotide point mutation (SNP) was discovered in the genome of *H. contortus* that was associated with BZ resistance (Kwa et al., 1994). This SNP occurs at location (P200) of the beta-tubulin isotype 1 gene, where the nucleobase thymine is

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replaced by adenine (TTC to TAC – F200Y). The presence of a homozygous resistant genotype has been stated to indicate the presence of AR (Elard et al., 1999). Although other BZ resistance associated SNPs have also been identified (i.e., TTC to TAC – F167Y, GAA to GCA – E198A) (Ghisi et al., 2007; Prichard, 2001; von Samson-Himmelstjerna et al., 2007), the conversion from TTC to TAC at the P200 location has been found to occur most frequently (Baltrušis et al., 2020a, 2020b; Silvestre and Cabaret, 2002; von Samson-Himmelstjerna et al., 2009).

A droplet digital PCR (ddPCR) assay for the detection and quantification of the F200Y SNP in *H. contortus* populations has been developed in Sweden and has proven to be a robust and accurate method (Baltrušis et al., 2018, 2020a, 2020b). In this method, the sample DNA (or RNA) in each well is partitioned into 20,000 nanodroplets, wherein a PCR reaction takes place in each droplet. Then, the droplet reader assesses all droplets (i.e., positive, or negative for the specific target of interest). By the Quantasoft software, results are automatically analysed by Poisson statistics; the number of positive and negative droplets are displayed, and the fractional abundance of the detected target(s) is presented. The high number of reactions in each sample/well (i.e., in each of the 20,000 droplets) provides a high sensitivity. Moreover, it has certain advantages over other PCR based methods, as no standards or references are required. The ddPCR has been shown to be particularly useful for sensitive detection of rare mutant alleles as it reduces background DNA competition (Hindson et al., 2011).

The aim of our study was to investigate the occurrence of BZ-resistant *H. contortus* in selected Norwegian sheep flocks using a previously published ddPCR assay (Baltrušis et al., 2018). In addition, we wanted to investigate whether there were any associations between the occurrence of resistance alleles and regional distribution.

2. Materials and methods

2.1. Selection of flocks

Sheep flocks were recruited from the Norwegian sheep recording system, with selection of flocks using data previously collected using a questionnaire (Gravdal et al., 2021). The dataset consisted of responses from 1378 sheep farmers (10% of Norwegian flocks) and included information from all counties in Norway. First, farmers were stratified by geographical location (i.e., western, eastern, central, and northern regions), and then a random selection from each area was made in Stata SE/16.0 (Stata Statistics/Data Analysis: Release 16. College Station, TX: StataCorp LLC). In addition, some flocks were recruited through communication with farmers (e.g., project presentations/meetings). The number of flocks selected from each area was based on the general flock population (Statistics, 2021), with the proportion of flocks included per region ranging from <1 to 2.4% of the total number of flocks in that area (Table 1).

Table 1
Geographical distribution of sheep flocks in Norway, and number and proportion included in the study. Columns 3, 4, 5 show the number of flocks (n) sampled in either both seasons (spring/ewes and autumn/lambs) or on only one occasion (ewes/spring or lambs/autumn).

Area (Counties*)	Flocks sampled/ total no of flocks (%)	Both seasons	Only spring [ewes]	Only autumn [lambs]
Northern (TF, N)	34/1399 (2.4)	24	10	
Central (T)	7/1194 (0.6)	7		
Eastern (I, OV, VT)	40/3118 (1.3)	30	9	1
Western (R, V, MR)	87/6977 (1.2)	70	16	1
Southern (A)	1/736 (0.1)	1		
Total n (%)	169/13424 (1.3)	132	35	2

* TF: Troms og Finnmark, N: Nordland, T: Trøndelag, I: Inland, OV: Oslo and Viken, VT: Vestfold and Telemark, R: Rogaland, V: Vestland, MR: Møre and Romsdal, A: Agder.

2.2. Sample collection

In 2021/2022, farmers of the selected flocks were requested to collect individual samples from 10 randomly selected ewes (spring i.e. April/May), and from 10 randomly selected lambs (autumn i.e. August/September). None of the animals sampled should have been treated with anthelminthics in the six weeks prior to sampling. The necessary equipment was provided by the Norwegian University of Life Sciences (NMBU); i.e., an information pamphlet, animal information sheets for completion (treatment/ear tag-identification numbers), sample bags, gloves, "faecal spoons", and a prepaid labelled envelope for shipping. Samples were taken directly from the rectum, either by hand or using the "faecal spoon". All samples were immediately placed in individual airtight ziplock-bags and sent overnight by post. After their arrival, the samples were processed promptly. The samples were stored in the refrigerator (4 °C) for a maximum of one to two days before processing.

1.1. Larval culture and collection

A total of four pooled cultures were made from each flock (i.e., two for ewes and two for lambs). Approximately 25 g of faeces (5 g from each of the 5 animals) were placed in plastic cups. After thorough mixing with a wooden spatula, water was added to obtain a thick paste. The cultures were incubated at 25 °C for two weeks. Every second day, the cultures were mixed, and enough water was added to keep the viscosity of the faecal paste remained approximately the same as on the day of preparation. Using the Baermann technique (Tintori et al., 2022), the larval suspension was collected into tubes (10 ml of each culture), which were centrifuged at 580*g* for 3 min. The supernatant was then aspirated, leaving about 1 ml of the suspension in the tube. The precipitated larvae were stored at −18 °C until molecular analysis.

2.3. Molecular analysis

2.3.1. DNA extraction from larvae

After thawing the larvae at room temperature, DNA was extracted from each of the pooled samples, using the Nucleospin DNA stool kit (Macherey-Nagel, PA, USA) in combination with 2 × 60 s of bead beating (FastPrep-24 5G, MP Biomedicals CA, USA). The method was applied according to the manufacturer's protocol, using 220 μl of larval suspension. DNA templates were frozen (−20 °C) until molecular analysis. DNA from positive controls was obtained by the same extraction procedure from adult *H. contortus* worms that were both resistant (TAC mutant) and susceptible (TTC wild type). These were kindly provided by Prof. Marián Várady, Institute of Parasitology, Slovak Academy of Sciences.

2.3.2. Digital droplet PCR (ddPCR)

The presence of *Haemonchus* in each of the pooled samples of larval DNA was determined by ddPCR, using primer/probe set targeting a *Haemonchus*-specific region in the internal transcribed spacer region 2 (ITS2) of the ribosomal RNA gene array (see Supplementary material for primer/probe sets used). Thereafter, duplex ddPCR discrimination assays were performed on the same samples, using primer/probe sets targeting the β-tubulin isotype 1 gene for the possible SNP at codon 200 (Table 2). The assay estimated the relative proportions of both TTC/TAC alleles simultaneously in *H. contortus* larvae in the sample population, using the previously published protocol (Baltrušis et al., 2018). The reaction mix consisted of 11 μl of ddPCR supermix (no dUTP) (BioRad), 1.1 μl of 20× stock solution of each of primer/probe mix (wildtype probe FAM, mutant probe HEX) and 6.8 μl of nuclease free water per well. RNase free water was used as a non-template control, while DNA from mutant (TAC) and susceptible (TTC) *H. contortus* were used as positive

Table 2
Primer/probe sets used in ddPCR assay.

	Susceptible	Mutant
Forward	TCGTGGAACCCTACAATGCT	TCGTGGAACCCTACAATGCT
Reverse	TCAAAGTGCGGAAGCAGATA	TCAAAGTGCGGAAGCAGATA
Probe	AACACCGATGAAACATTCTGTATTGAC	AACACCGATGAAACATACTGTATTGAC
Fluorophore	FAM	HEX

Overview of primer/probe sequences used (Baltrušis et al., 2018; Redman et al., 2015).

controls for each of the respective targets. Droplet formation and thermocycling were then performed as per manufacturer's instructions (BioRad). After amplification, the PCR plate was placed into the droplet reader (QX200) (BioRad) and analysed using Quantasoft™ software (BioRad). Oversaturated samples were diluted by diluting 20 times in RNase free water and the sample was analysed again.

2.4. Statistical analysis

After ddPCR, all results were analysed for the presence of positive droplets using Poisson statistics with the Quantasoft software (BioRad). Thresholds were manually adjusted according to the baselines of the negative and positive controls (FAM: 4000, HEX: 3000). Samples that had >2.5 copies per μl were classified as positive for the respective targets, in agreement with the limit of detection (LoD) of the ddPCR assay (Baltrušis et al., 2018). In detail; for determining the presence of *Haemonchus* in lambs and ewes, the respective age group were classified as positive if one or both pooled samples were positive (i.e., > 2.5 copies per μl). Furthermore, flocks were classified as *Haemonchus* positive if *Haemonchus* was detected in either one or both age groups. Similarly, samples from lambs and ewes were classified as positive for the respective target (i.e., TTC/TAC), if it was detected (> 2.5 copies per μl) in either one or both pooled samples. Descriptive statistical analysis was then performed using Excel and Stata SE/16.0 (Stata Statistics/Data Analysis: Release 16. College Station, TX: StataCorp LLC). Pearson chi squared and Fisher's exact tests were used to compare differences of categorical variables, *Haemonchus* detected in lambs and ewes, and by sampling area. Maps were created using the choropleth map (Fig. 1 a), and symbol map (Fig. 1 b, c, and Fig. 2) in Datawrapper.

3. Results

3.1. General demographics

Faecal samples were collected from a total of 169 sheep flocks, from all regions of Norway (see Fig. 1 a). Of these, 67% (113/169) were randomly selected from the questionnaire data set, while 33% (56/169) of the flocks were added through communication with farmers. Just one flock from the Southern region was included, as this area was not originally part of the scope of our study. A total of 132 flocks were sampled in both spring (ewes) and autumn (lambs) (132/169), while 20% (35/169) were sampled only in spring (ewes), and two flocks were sampled only in autumn (lambs) (2/169) (Table 1).

3.2. ddPCR

3.2.1. Occurrence of Haemonchus

Haemonchus was detected in 70% (118 /169) of the total flocks examined by ddPCR (see Fig. 1 b). The frequency of detection on flock level was similar in both age groups, i.e. ewes (63%) and lambs (60%). Geographical differences were evident ($p < 0.001$), with a lower occurrence in the northern region (Fig. 1 c) in both lambs (8%) and ewes (21%). The occurrence gradually increased from north to south, with 71% in lambs and 72% in ewes in the eastern region, and highest values in the western region in both lambs (75%) and ewes (79%) (Table 3).

3.2.2. Occurrence of mutant type H. contortus

Among flocks with *Haemonchus*, the mutant type (TAC) of *H. contortus* was detected in the majority of them, both in lambs (73%) and ewes (69%). In contrast, the wild type (TTC) was only detected in 5% of the flocks. However, regional differences were distinguishable with the highest occurrence of wild type found in ewes in the northern region (57%) (Table 3, Fig. 2).

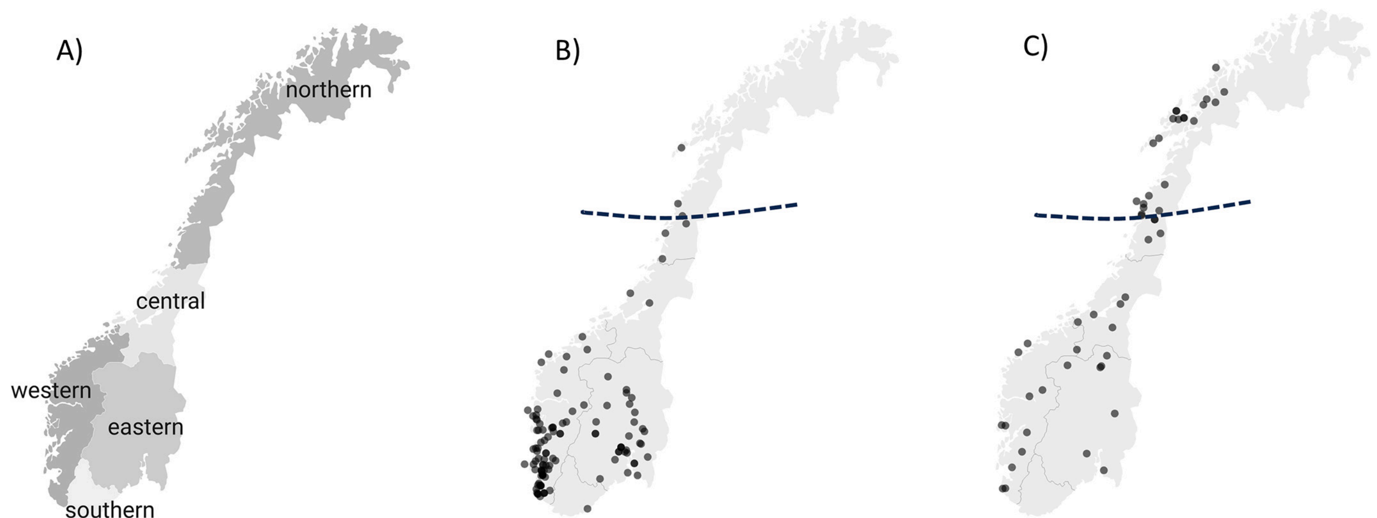

Fig. 1. Overview of; A) regions in Norway, B) the geographical distribution of flocks where *Haemonchus* were present ($n = 118$), and C) not present ($n = 51$). The Arctic circle (66° 34′N) is indicated by the stapled line.

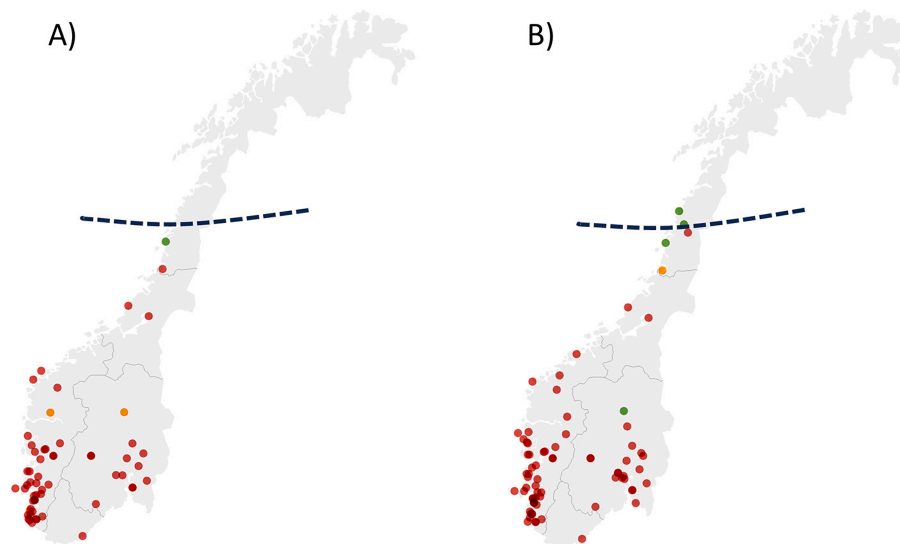

Fig. 2. Geographical distribution of the occurrence of; mutant type TAC (red dots), wild type TTC (green dots), and mix of both (orange dots), detected in samples from lambs (A, $n = 59$) and ewes (B, $n = 77$). Flocks with TAC/TTC concentration < 2.5 copies per μl are not included. The Arctic circle (66° 34′N) is indicated by the stapled line. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 3
Occurrence of *Haemonchus* at flock level, and the proportion of those flocks where the mutant (F200Y, TAC) type and wild (F200, TTC) type were detected.

Area	Age group* (number of flocks)	*Haemonchus* Number of flocks (%)	F200Y (TAC)** Number of flocks (%)	F200 (TTC)** Number of flocks (%)
Northern	Lambs (24)	2 (8)	1 (50)	1 (50)
	Ewes (34)	7 (21)	2 (29)	4 (57)
Central	Lambs (7)	2 (29)	2 (100)	0
	Ewes (7)	2 (29)	2 (100)	0
Eastern	Lambs (31)	22 (71)	14 (64)	1 (5)
	Ewes (39)	28 (72)	20 (71)	1 (4)
Western	Lambs (71)	53 (75)	40 (75)	2 (4)
	Ewes (86)	68 (79)	48 (71)	0
Southern	Lambs (1)	1 (100)	1 (100)	0
	Ewes (1)	1 (100)	1 (100)	0
Total	Lambs (134)	80 (60)	58 (73)	4 (5)
	Ewes (167)	106 (63)	73 (69)	5 (5)

* Since samples were not attained both seasons from all flocks, the number (n) of flocks varies between age groups.
** Flocks with TAC/TTC concentration < 2.5 copies per μl are not included.

4. Discussion

In this study, we performed a ddPCR screening to determine the occurrence of a mutation in *H. contortus* associated with BZ resistance. To reveal the regional distribution pooled samples of ewes and lambs were collected from sheep flocks throughout Norway. In general, we observed a north-south gradient in the occurrence of F200Y. To our knowledge, this is the first report of the occurrence of the F200Y (TAC) mutation in *H. contortus* in sheep in Norway, detected by ddPCR or any other molecular method.

Although *H. contortus* is known to thrive in warmer, humid climates (Besier et al., 2016), it has previously also been found in northern parts of Norway, more specifically in the Lofoten archipelago located north of the Arctic circle in Nordland County (latitude: 68.15) (Domke et al., 2013). This also seems to be the case in other northern countries such as Sweden and Canada, which are at approximately the same latitudes as Norway (Borkowski et al., 2020; Halvarsson and Höglund, 2021; Queiroz et al., 2020a). This can be explained by the adaptation of the parasite to survive within hosts during the winter season as hypobiotic larvae (Troell et al., 2005). The regional differences in the occurrence of *Haemonchus* are likely linked to multiple factors, such as latitude, altitude, and climatic conditions, of which large variations can be found both between and within regions of Norway.

Overall, our results suggests that the F200Y (TAC) mutation is common in Norwegian sheep flocks. Similar results have been reported from Sweden (Baltrušis et al., 2020a, 2020b). This could be related to the prolific nature and the high genetic variability of *H. contortus*, which are excellent traits for rapidly development of AR (Doyle et al., 2020). Interestingly, the wild type F200 (TTC) was detected more frequently in the north than the mutant type F200Y (TAC). This is probably related to less-intensive treatment regimes in this region (Domke et al., 2011) and thus less selection pressure on the parasites.

The FECRT study performed in 2008–2009 indicated BZ resistance in 10 to 31% of flocks (reaching 80% in Rogaland County) with *Teladorsagia/Trichostrongylus* as main larval type recovered followed by *Haemonchus* (Domke et al., 2012). Although comparison is limited due to the different method used in the studies, our findings show that the mutant variant of *Haemonchus* (F200Y/TAC) is widespread in Norwegian sheep flocks, and that ddPCR is a useful tool for large-scale screening of this SNP. Nevertheless, other molecular methods could have been utilized, such as allele specific PCR or metabarcoding (Halvarsson and Höglund, 2021; Queiroz et al., 2020b; Redman et al., 2019). While ddPCR can detect only a limited set of species/genera, the nemabiome sequencing platform allow determination of the total species composition within hosts (Avramenko et al., 2015).

In the present study, there could be a selection bias, as only those farmers who completed the questionnaire and agreed to participate in the project were included. Nonetheless, to obtain samples from their respective flocks, consent was pivotal.

Some pooled faecal samples for larval culture weighed <25 g because the material in individual samples was limited. However, previous studies have found that the number of larvae may be of limited importance, due to the high sensitivity of the ddPCR (Baltrušis et al., 2020a, 2020b).

The thresholds for channel 1 (FAM) and channel 2 (HEX) positive droplets were set to a lower amplitude of fluorescence than previously described (Baltrušis et al., 2018). This was based on coherence with the negative control as baseline, the positive controls for both targets, and the optimal separation between the bands of negative and positive droplets. The cause of this amplitude variation is unknown, but might be explained by differences in technical equipment.

In this study, we investigated one of the three known mutations implicated in BZ-resistance. We focused on the F200Y (TAC) SNP, as this is the most common BZ-resistance-mutation worldwide, including in Europe (Baltrušis et al., 2020a, 2020b; Chaudhry et al., 2015; Claerebout et al., 2020). The ddPCR analysis is not currently available for use in routine diagnostic laboratories in Norway. However, it is a valuable tool for research and for cases where high sensitivity and/or specificity is required.

5. Conclusion

This study shows that, *H. contortus* is common in Norwegian sheep flocks, both in lambs (60%) and ewes (63%). Regional differences were found, with the nematode most frequently detected in flocks in western and eastern areas. Moreover, the mutant variant (F200Y/TAC) of *H. contortus*, associated with BZ-resistance, was detected in most of these flocks and showed widespread distribution, while the wild/susceptible type of *H. contortus* is relatively rare in Norwegian sheep flocks.

Ethical statement

Hereby, I Maiken Gravdal consciously assure that for the manuscript Benzimidazole-resistance associated mutation in *Haemonchus contortus* in Norwegian sheep, as detected by droplet digital PCR the following is fulfilled:

1) This material is the authors' own original work, which has not been previously published elsewhere.
2) The paper is not currently being considered for publication elsewhere.
3) The paper reflects the authors' own research and analysis in a truthful and complete manner.
4) The paper properly credits the meaningful contributions of co-authors and co-researchers.
5) The results are appropriately placed in the context of prior and existing research.
6) All sources used are properly disclosed (correct citation). Literally copying of text must be indicated as such by using quotation marks and giving proper reference.
7) All authors have been personally and actively involved in substantial work leading to the paper, and will take public responsibility for its content.

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Declaration of Competing Interest

None.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.vprsr.2023.100938.

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